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<b>(21) International Application Number:</b> PCT/US91/02551 <b>(22) International Filing Date:</b> 10 April 1991 (10.04.91) <b>(30) Priority data:</b> 507,939 11 April 1990 (11.04.90) US <b>(60) Parent Application or Grant</b> <b>(63) Related by Continuation</b> US 507,939 (CON) Filed on 11 April 1990 (11.04.90) <b>(71)(72) Applicant and Inventor:</b> PARIKH, Indu [US/US]; 2558 Booker Creek Road, Chapel Hill, NC 27514 (US). <b>ERREUR CODE DEPOSANT BFG:</b> SIEGEL, Robert, S. ; 4360 Huggins Street, San Diego, CA 92122 (US). WON- DRACK, Lillian, M. ; 28 Corey Lane, Niantic, CT 06357 (US). HUTTON, James, R. ; North 69, West 272711, Hickory Chasm Road, Sussex, WI 53089 (US). AMAR- ANT, Tanchum ; 68 Rambam St., 76 100 Rehovot (IL). GROSS, Roger, W. ; 10953 West Langlaee, Milwaukee, WI 53225 (US).	<b>(74) Agents:</b> EHRLINGER, David, B. et al.; Krass & Young, 3001 W. Big Beaver, Suite 624, Troy, MI 48084 (US). <b>(81) Designated States:</b> AT (European patent), AU, BB, BE (European patent), BG, BR, CA, CH (European patent), DE (European patent), DK (European patent), ES (Eu- ropean patent), FI, FR (European patent), GB (Euro- pean patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU (European patent), MC, MG, MW, NL (European patent), NO, RO, SD, SE (Eu- ropean patent), SU, US.  <b>Published</b> <i>With international search report.</i> <i>With amended claims.</i>	
<b>(54) Title:</b> METHOD FOR THE PURIFICATION OF EPIDERMAL GROWTH FACTOR  <b>(57) Abstract</b>  Method for the recovery and purification of epidermal growth factor peptides and large volumes of EGF-containing medi- um are described, comprising a series of adsorption-desorption steps employing specified adsorbents, followed by high perform- ance liquid chromatography. Product EGF peptides are highly purified and suitable for use in a variety of clinical applications.		

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METHOD FOR THE PURIFICATION OF EPIDERMAL GROWTH FACTOR

This invention relates to purification methods. In a particular aspect, this invention relates to the purification of epidermal growth factor peptides from fluid medium containing same. In one aspect, the present  
5 invention relates to methods for purification of epidermal growth factor peptides produced by recombinant techniques. In another aspect, the present invention relates to methods for the purification of epidermal growth factor peptides produced by yeast cells transformed with at least one copy  
10 of a DNA sequence encoding an epidermal growth factor peptide.

BACKGROUND OF THE INVENTION

Epidermal growth factor (EGF; formerly also known as urogastrone) is a regulatory peptide having multiple  
15 biological activities. For example, EGF causes complete inhibition of gastric acid secretion within about 15 minutes of administration. In addition, application of EGF over a longer time period stimulates epidermal tissue growth. Such physiological effects make EGF a candidate  
20 for various clinical applications. Applications for which EGF is currently being tested include corneal transplant healing, skin graft donor sites, diabetic ulcers, gastrointestinal ulcers, and the like. Clearly the widespread use of EGF in such clinical applications will  
25 require the development of efficient production and purification systems.

EGF was first isolated and characterized from

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murine submaxillary gland tissue. Human EGF ( $\beta$ -urogastrone) isolated from human urine is a 53 amino acid peptide containing three disulfide bonds. The availability of the amino acid sequence of  $\beta$ -urogastrone allowed the design and construction of synthetic genes encoding this peptide. The availability of synthetic genes enabled the development of recombinant expression systems for the production of this peptide.

The first reported recombinant expression system for the production of human EGF utilized E. coli and yielded 2.3 mg/L of biologically active material. Cytoplasmic expression of human EGF in S. cerevisiae reached a level of about 30 micrograms per liter, but the product contained an N-terminal methionine. Later, the use of the S. cerevisiae  $\alpha$ -mating factor leader sequence to direct secretion of human EGF from S. cerevisiae increased the expression level of a (1-52) form of human EGF to about 5 milligrams per liter. More recently, improved Bacillus expression hosts have been reported which are capable of secreting up to 240 milligrams per liter EGF, with no appreciable degradation. With the exception of the Bacillus expression system, for which no published information on EGF productivity during large scale production is available, expression levels of human EGF in recombinant expression systems has been quite low. Furthermore, none of the publications which describe recombinant production of EGF address the problem of EGF recovered from the medium in which the peptide is prepared.

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The methylotrophic yeast Pichia Pastoris has recently been developed as an improved host for the production of recombinant products. Recombinant Pichia pastoris strains have been shown to be capable of secreting  
5 recombinant proteins in the gram per liter range. In addition, such strains have been shown to be capable of adapting to fed batch or continuous cultivation fermentation conditions. Moreover, such strains have an extremely stable recombinant phenotype and are capable of  
10 maintaining high yields of the desired recombinant expression product over several orders of fermentation scale. Indeed, Siegel, et al., in copending application Serial No. 323,964, filed March 15, 1989, have recently shown that P. pastoris is an excellent host for the  
15 recombinant production of EGF. The disclosure of this copending application is hereby incorporated by reference in its entirety. In view of the availability of medium containing high levels of recombinantly produced EGF, there is needed an efficient means for the recovery and  
20 purification of EGF from such medium.

#### STATEMENT OF THE INVENTION

In accordance with the present invention, we have developed an efficient method for the recovery and purification of EGF peptides from fluid medium containing  
25 same. The invention method involves successive adsorption-desorption on appropriate resin materials, followed by reverse phase high performance liquid chromat graphy,

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followed by optional filtration and lyophilization steps. In this way, greater than one gram of purified EGF can be obtained per 10 liters of fermentation broth. Typically, greater than about 55% recovery of the EGF initially  
5 present in the crude EGF-containing medium can be achieved by the invention process.

#### BRIEF DESCRIPTION OF THE FIGURE

Figure 1 is a restriction map of plasmid pEGF819, which contains five human EGF expression cassettes.

#### 10 DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there is provided a method for the purification of epidermal growth factor (EGF) peptides from medium containing EGF, said method comprising:

15 (a) contacting said medium with a sufficient quantity of a reverse phase resin and under conditions suitable to adsorb at least 90% of said EGF from said medium,

(b) eluting the adsorbed EGF from said EGF-  
20 containing resin of step (a) by contacting said resin with a sufficient quantity of a solvent system which is sufficiently non-polar so as to displace substantially all of said EGF from said resin,

(c) contacting the eluate from step (b) with a  
25 sufficient quantity of a cation exchange resin and under conditions suitable to adsorb at least 95% of said EGF from

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said eluate,

(d) eluting the adsorbed EGF from said cation exchange resin by contacting said resin with at least 1.5 volumes, relative to the volume of resin, of a buffer system having a sufficient ionic strength so as to displace substantially all of said EGF from said resin, and thereafter

(e) subjecting the eluted EGF obtained from step (d) to preparative-scale high performance liquid chromatography (HPLC).

In accordance with a specific embodiment of the present invention, there is provided a method for the purification of human epidermal growth factor (hEGF) peptides from medium containing hEGF, wherein said medium containing hEGF is the fermentation broth from a high cell density yeast fermentation operation, and wherein said yeast are transformed with at least one DNA fragment capable of expressing hEGF, said method comprising:

(a) contacting said medium with a sufficient quantity of a reverse phase resin and under conditions suitable to adsorb at least 90% of said hEGF from said medium; wherein said reverse phase resin is C<sub>18</sub>-type resin; wherein at least 30 grams, per gram of hEGF in said medium, of said reverse phase resin are employed; and wherein said contacting is carried out for a time in the range of about 0.1 up to 8 hours, at a temperature in the range of about 4 up to 40°C;

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(b) separating the hEGF-containing resin from the hEGF-depleted medium,

(c) contacting the hEGF-containing resin with at least 1 volume, per volume of resin, of a dilute, weak acid; wherein the dilute, weak acid is a 0.05 M acetic acid solution, then removing the dilute, weak acid from the hEGF-containing resin,

(d) eluting the adsorbed hEGF from said hEGF-containing resin of step (c) by contacting said resin with a sufficient quantity of a solvent system which is sufficiently non-polar so as to displace substantially all of said hEGF from said resin,

(e) contacting the eluate from step (d) with a sufficient quantity of a cation exchange resin and under conditions suitable to adsorb at least 95% of said hEGF from said eluate,

(f) contacting the hEGF-containing resin produced in step (e) with sufficient quantity of a dilute, weak acid solution to reduce the absorbance, at 400 nanometers, of the effluent from said contacting to 0.1 A.U. or less, relative to a blank sample of the dilute, weak acid solution,

(g) eluting the adsorbed hEGF from said cation exchange resin by contacting said resin within the range of about 1.5 up to 3 volumes, relative to the volume of resin, of a buffer system having an ionic strength of about 0.3 g-ions/L, and comprising 0.3 M ammonium acetate,

(h) adjusting the pH of the eluted hEGF



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obtained from step (g) by the addition thereto of a sufficient quantity of trifluoroacetic acid to render said solution about 0.1% in TFA,

(i) loading the eluted, pH-adjusted hEGF  
5 obtained from step (h) onto a preparative-scale high performance liquid chromatography column (HPLC),

(j) initially treating the loaded column within the range of 1-2 column volumes of a solvent system which is sufficiently non-polar to elute impurities less  
10 hydrophobic than hEGF, but not so non-polar as to cause elution of significant amounts of hEGF, and thereafter

(k) eluting purified hEGF from said HPLC employing a mixed solvent system comprising an aqueous component and an organic component, wherein said mixed  
15 solvent system is sufficiently non-polar to elute hEGF, but not so non-polar as to cause elution of significant quantities of materials which are more tightly bound to the HPLC support than is hEGF.

The term "epidermal growth factor" or "EGF  
20 peptide", as used throughout the specification and in the claims, refers to a polypeptide product which exhibits similar, in-kind, biological activities to natural human epidermal growth factor (hEGF), as measured in recognized bioassays, and has substantially the same amino acid  
25 sequence as hEGF, including the 53, 52 and 48 amino acid forms. It will be understood that polypeptides deficient in one or more amino acids in the amino acid sequence reported in the literature for naturally occurring hEGF, or

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polypeptides containing additional amino acids or polypeptides in which one or more amino acids in the amino acid sequence of natural hEGF are replaced by other amino acids are within the scope of the invention, provided that

5 they exhibit the functional activity of hEGF, e.g., inhibition of the secretion of gastric acid and promotion of cell growth. The invention is intended to embrace all the allelic variations of hEGF. Moreover, derivatives obtained by simple modification of the amino acid sequence

10 of the naturally occurring product, e.g., by way of site-directed mutagenesis or other standard procedures, are included within the scope of the present invention. EGF forms produced by proteolysis of host cells that exhibit similar biological activities to mature, naturally

15 occurring hEGF are also encompassed by the present invention.

The first step in the invention purification process is to contact epidermal growth factor-containing medium with a sufficient quantity of a reverse phase resin

20 and under conditions suitable to adsorb at least 90% of the epidermal growth factor from the medium.

Contacting of the epidermal growth factor-containing medium with reverse phase resin can be carried out in a variety of ways. For example, the reverse phase

25 resin can be contained in a column through which the epidermal growth factor-containing medium is percolated. Alternatively, the reverse phase resin can be contained in a closed vessel into which the epidermal growth factor-

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containing medium is introduced, followed by stirring for a sufficient period of time to allow there to occur the rough contacting of the resin and fluid medium, followed by decanting of the EGF-depleted medium from the EGF-  
5 containing reverse phase resin. As another alternative, the reverse phase resin (which has first been wetted with a suitable, water soluble solvent such as methanol or acetonitrile) can be added to the vessel containing the EGF-containing medium, followed by removal of EGF-depleted  
10 medium from the EGF-containing reverse phase resin.

Reverse phase resins contemplated for use in the practice of the present invention are well known in the art and include  $C_8$ - $C_{18}$  resins, CN (cyano) resins,  $NH_2$  (amino) resins, phenyl resins, and the like. See, for example,  
15 Melande and Horvath at pp. 114-319 (especially pp. 123-165) of High Performance Liquid Chromatography, Advances and Perspectives, Vol. 2, C. Horvath, ed. Academic Press (New York, 1980). Presently preferred reverse phase resins for use in the practice of the present invention are  $C_{18}$ -type  
20 resins.

The quantity of reverse phase resin employed can vary widely. Typically, at least about 30 grams, per gram of EGF contained in the medium, of reverse phase resin, will be employed.

25 Contacting of EGF-containing medium with reverse phase resin can be carried out under a variety of conditions. Typically such contacting is carried out for a time in the range of about 0.1 up to 8 hours, and at a

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temperature in the range of about 4 up to 40°C.

Once EGF-containing broth has been maintained in contact with reverse phase resin for a period of time sufficient for EGF to adsorb to the resin, it is desirable  
5 to remove the EGF-depleted medium from contact with the EGF-rich reverse phase resin. This can be accomplished in a variety of ways such as, for example, filtration, decantation, centrifugation, and the like. This contacting and separation can readily be accomplished in one  
10 operational step by passing the medium containing EGF through a column of the reverse phase resin, wherein the column is equipped with a retaining means (e.g., a screen, support plate with holes, or the like), so as to retain the reverse phase resin in the column, yet allow fluid medium  
15 to pass therethrough. In this way, the EGF-depleted broth is allowed to merely percolate through the reverse phase resin.

Once substantially all of the EGF has been adsorbed onto the reverse phase resin, and prior to elution  
20 of the EGF therefrom, it is desirable to contact the EGF-containing resin with in the range of about 1 up to 10 volumes, relative to the volume of the reverse phase resin, of a dilute, weak acid; and thereafter, the dilute, weak acid is then removed from the EGF-containing resin. This  
25 optional wash serves to remove impurities which are not as tightly bound to the reverse phase resin as is the EGF. Exemplary dilute, weak acid include approximately 0.05 molar acetic acid, formic acid, or phosphoric acid

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solutions.

Once substantially all of the EGF has been adsorbed onto the reverse phase resin and optionally rinsed as described above, EGF is then eluted from the EGF-  
5 containing resin by contacting the resin with a sufficient quantity of a solvent system which is sufficiently non-polar so as to displace substantially all of the EGF from the resin. A solvent system which is "sufficiently non-polar" to accomplish the desired elution is a solvent  
10 system which is less polar than the aqueous medium from which the EGF is being recovered. Elution is accomplished by reducing the polarity of the solvent system, typically by the addition of organic solvents thereto. A solvent system which is "sufficiently non-polar" to accomplish the  
15 desired elution is one wherein the polarity has been sufficiently reduced so as to substantially increase the partitioning of EGF peptides into the mobile phase from the stationary phase.

Solvents contemplated for use in this elution  
20 step include aqueous alcohol mixtures containing at least one alcohol having up to four carbon atoms, aqueous acetonitrile solutions, ketones having up to six carbon atoms, cyclic ethers or cyclic polyethers having up to six carbon atoms, and the like. Presently preferred solvent  
25 systems for use in this elution step include (1) about 38% aqueous ethanol, or (2) 30% aqueous acetonitrile.

The quantity of the eluting solvent system employed can vary widely. Typically, in the range of about

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2-4 volumes of the solvent system, per volume of reverse phase resin, will be employed.

Elution of EGF from the EGF-containing reverse phase resin can be carried out under a variety of conditions. Typically, a temperature in the range of about 10 up to 40°C will be employed. Typically, elution time will be relatively short, falling in the range of about 0.1 up to 1.0 hour, although longer or shorter times can also be employed.

Partially purified EGF-containing medium which has been eluted from the reverse phase resin is then contacted with a sufficient quantity of a cation exchange resin and under conditions suitable to adsorb at least 95% of the EGF from the EGF-containing medium.

Cation exchange resins contemplated for use in the practice of the present invention include carboxymethyl cellulose, carboxymethyl sephadex, sulphopropyl cellulose, sulphopropyl sephadex, and the like. Presently preferred cation exchange resins include carboxymethyl cellulose and carboxymethyl sephadex, with carboxymethyl cellulose being the presently most preferred cation exchange resin for use in the practice of the present invention because of its ready availability and excellent performance.

Quantities of cation exchange resin employed in the practice of the present invention can vary widely. Typically, in the range of about 0.25 up to 1 liter of cation exchange resin per gram of EGF in the medium being treated will be employed.

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Contacting of partially purified EGF-containing broth with cation exchange resin can be carried out under a variety of conditions. Typically, such contacting is carried out for a time of at least one minute and a  
5 temperature in the range of about 4 up to 40°C.

Once substantially all of the EGF has been adsorbed by the cation exchange resin, the EGF-containing resin is optionally contacted with a sufficient quantity of a dilute, weak acid solution so as to reduce the  
10 absorbance, at 400 nanometers, of the effluent from the contacting to 0.1 A.U. or less, relative to a blank sample of the dilute, weak acid solution. Presently preferred weak acid solutions for use in this rinsing step include approximately 0.05 molar acetic acid, formic acid or  
15 phosphoric acid solutions.

Once substantially all the EGF has been adsorbed onto the cation exchange resin and optionally rinsed with dilute, weak acid, the EGF is eluted from the cation exchange resin by contacting the resin with at least 1.5  
20 volumes, relative to the volume of the resin, of a buffer system having a sufficient ionic strength so as to displace substantially all of the EGF from the resin. Buffer systems contemplated for use in the practice of the present invention are those having an ionic strength of at least  
25 about 0.1 g-ions per liter, and are typically selected from ammonium acetate, ammonium formate, sodium acetate, potassium acetate, sodium chloride, and the like.

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Presently preferred buffer systems useful in the practice of the present invention are those having an ionic strength of at least about 0.3 g-ions per liter. An especially preferred buffer system is a 0.3 molar ammonium acetate  
5 solution.

The quantity of high ionic strength buffer system employed in the practice of the present invention can vary widely. Typically, quantities of buffer falling in the range of about 1.5 up to 3 volumes, per volume of cation  
10 exchange resin, will be employed.

Elution of EGF from cation exchange column can be carried out at a variety of temperatures. Typically, temperatures in the range of about up to 40°C are employed.

Cation exchange resin can be optionally activated  
15 prior to use and/or regenerated after EGF elution, by treating according to regeneration techniques known in the art, as typically provided by resin manufacturers. For example, resin can first be contacted with a concentrated salt solution to cause elution of most materials adsorbed  
20 thereon, then washed with a dilute solution of a strong base, then equilibrated with a dilute, weak acid. The column so treated is then ready for contacting with the partially purified EGF-containing broth as described above.

Prior to loading the eluted EGF-containing  
25 solution onto a high performance liquid chromatography (HPLC) column, the EGF-containing solution can optionally be treated with a sufficient quantity of trifluoroacetic acid so to render the EGF-containing solution about 0.1% in



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trifluoroacetic acid.

Once the EGF-containing solution has been eluted from the cation exchange resin, and optionally adjusted to be about 0.1% in trifluoroacetic acid, at least a portion  
5 of this solution is then subjected to high performance liquid chromatography.

Columns contemplated for use in this HPLC step are well known in the art and include C<sub>8</sub>-C<sub>18</sub> resins, CN (cyano) resins, NH<sub>2</sub> (amino) resins, phenyl resin, and the  
10 like. See, for example, Melander and Horvath, supra. Presently preferred HPLC columns for use in this step of the invention process include C<sub>18</sub>-type resins. A presently preferred column configuration is a two-inch diameter radial compression column.

15 The loading capacity of various HPLC column packings can vary widely. Any loading level up to about 50% of the breakthrough value for a given column packing and configuration is suitable. The breakthrough value for a given column occurs when the resin is saturated with  
20 adsorbent, such that introduction of any additional adsorbent to the system will either result in the failure of newly introduced material to adsorb to the resin, or in the immediate displacement of previously adsorbed material (to provide a site for adsorption of the newly introduced  
25 material). With the presently preferred C<sub>18</sub>-type resins, loading levels of up to about 14mg of EGF per cubic centimeter of column packing can be achieved.

Once loading of the HPLC-column with EGF-

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containing solution has been completed, the column is typically rinsed, prior to elution of EGF, by passing through the HPLC column in the range of about 1 up to 2 column volumes of an initial solvent system which is sufficiently non-polar to cause impurities which are less hydrophobic than EGF to elute, but which solvent system is not so non-polar as to cause elution of significant quantities of EGF. An exemplary solvent system employed for this initial HPLC treating step comprises (i) a 0.1% trifluoroacetic acid-containing aqueous solution, and optionally, (ii) up to 15% of a 95% acetonitrile-5% water mixture which contains 0.1% trifluoroacetic acetic acid.

In order to elute purified EGF from the HPLC column, a mixed solvent system is then passed through the HPLC column. Mixed solvent systems employed for this purpose comprise an aqueous component and an organic component, wherein the mixed solvent system is sufficiently non-polar to cause elution of EGF from the column, but not so non-polar as to cause elution of significant quantities of materials which are more tightly bound to the HPLC support than is EGF. It is preferred that EGF be gently displaced from the HPLC column, and this is typically accomplished by gradually increasing the proportion of the organic component of the mixed solvent system, relative to the proportion of the aqueous component of the mixed solvent system, during the course of the elution.

Typical solvent systems employed for elution of EGF from the HPLC column comprise an initial concentration

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of up to 100%, down to a final concentration as low as about 50% of a 0.1% trifluoroacetic acetic acid-containing aqueous solution, and an initial concentration as low as 0%, up to a final concentration of up to about 50%, of an  
5 aqueous acetonitrile mixture containing 0.1% trifluoroacetic acetic acid, wherein the aqueous acetonitrile contains up to 20% water.

Since acetonitrile is a commonly used solvent for elution of purified EGF from HPLC, and many applications of  
10 EGF require material which meets all Federal Drug Administration regulations for generally regarded as safe (GRAS) products, an additional adsorption-desorption step employing cation exchange resin can be employed to remove substantially all of the acetonitrile introduced in the  
15 previous steps. Thus, the products from the HPLC step can optionally be further subjected to

(f) contacting the eluate from step (e) with a sufficient quantity of a cation exchange resin and under conditions suitable to adsorb at least 95% of said  
20 EGF from said eluate,

(g) contacting the EGF-containing resin produced in step (f) with sufficient quantity of a dilute, weak acid solution to reduce the concentration of acetonitrile in the effluent to no greater than about 10  
25 mg/L, and

(h) eluting the adsorbed EGF from said cation exchange resin by contacting said resin with at least 1.5 volumes, relative to the volume of resin, of a

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buffer system having a sufficient ionic strength so as to displace substantially all of said EGF from said resin.

Cation exchange resins contemplated for use in this acetonitrile-removal step include carboxymethyl  
5 cellulose, carboxymethyl sephadex, sulphopropyl cellulose, sulphopropyl sephadex, and the like. Presently preferred cation exchange resins include carboxymethyl cellulose.

Cation exchange resins employed in this embodiment of the present invention can optionally be  
10 activated prior to use, and/or regenerated after EGF elution, as described above.

Dilute, weak acid solutions contemplated for use in this aspect of the present invention include approximately 0.05 molar acetic acid, formic acid, or  
15 phosphoric acid solutions, and the like.

Buffer systems contemplated for use in eluting the highly purified EGF from the EGF-containing cation exchange resin include buffer systems having an ionic strength of at least about 0.1 g-ions per liter, and are  
20 typically selected from ammonium acetate, ammonium formate, sodium acetate, potassium acetate, sodium chloride, and the like. Presently preferred buffer systems useful in the practice of the present invention are those having an ionic strength of at least about 0.3 g-ions per liter. An  
25 especially preferred buffer system is a 0.3 molar ammonium acetate solution.

A convenient form in which to store and transport purified EGF is in lyophilized form. Material eluted from

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the HPLC employing buffer systems comprised of organic salts can either be lyophilized directly, or after acetonitrile removal as described above, or after sterilization, which can be carried out in standard manner.

- 5 Material eluted from the HPLC employing buffer systems comprised of organic salts is typically treated by sterile filtration, then stored as bulk concentrate.

A presently preferred means for sterilizing the EGF-containing solutions is to pass the material through a  
10 pore filter having a pore size no greater than about 0.45 microns. Those of skill in the art can readily identify numerous other means by which the EGF-containing solutions can be sterilized.

The medium from which EGF is recovered according  
15 to the invention method for EGF purification can vary widely. The recovery of both natural and synthetic materials is presently contemplated. Due to their substantial similarity, EGF-like materials such as native human epidermal growth factor (1-53 hEGF), (1-48) analog of  
20 hEGF, (1-51) analog of hEGF, (1-52) analog of hEGF, as well as peptides which are substantially homologous thereto, are all contemplated for use in the practice of the present invention.

Synthetic sources of EGF from which EGF can be  
25 recovered and purified in accordance with the present invention include recombinant modified yeast and/or bacteria containing one or more DNA sequences operably encoding EGF peptides. Presently preferred are yeast

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species selected from the genus Pichia. The specific Pichia pastoris strains G+EGF817S1, G+EGF819S4 or G+EGF206S10 are presently more preferred because they have proven to produce high levels of EGF in large scale fermentation operation. These specific presently most preferred strains are prepared and caused to express EGF as described in copending application Serial No. 323,964, to which application the reader is directed for additional detail as to the preparation of the strains and expression of EGF therefrom.

When the EGF to be purified is contained in the fermentation broth from a fermentation operation, it is preferred to separate cellular and particulate material from the fermentation broth prior to the initial contacting of the EGF-containing medium with reverse phase resin.

The invention will now be described in greater detail with reference to the following non-limiting examples.

#### EXAMPLES

##### Example 1: EGF Production Strain

The strain of P. pastoris employed in the production of hEGF which contained four copies of a methanol-regulated hEGF expression cassette (a single expression cassette was comprised of the methanol-regulated P. pastoris AOX1 gene promoter, the S. cerevisiae  $\alpha$ -mating factor secretion sequences, a synthetic gene coding for

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either hEGF (1-53) or (1-48), the AOX1 transcription terminator, and the P. pastoris HIS4 gene for selection integrated into the host genome, was prepared as follows:

The auxotrophic His Pichia pastoris host strain  
5 GS115 (ATCC 20864) was transformed with a vector containing five hEGF expression cassettes. The vector comprised of five hEGF (1-53) expression cassettes is referred to as pEGF819, the preparation of which has been described in copending application Serial No. 323,964. A restriction  
10 map of plasmic pEGF819 is set forth in Figure 1.

Pichia pastoris strain GS115 was the host for transformation with this vector. The vector was linearized prior to transformation into GS115 by the spheroplast method [Cregg, et al., Mol. Cell. Biol. 5, 3376-3385  
15 (1985)]. After selection and analysis by Southern hybridization, strain G+EGF819S4 was found to contain four copies of the hEGF (1-53)-encoding cassette (one copy was apparently lost from the five-copy plasmid vector by recombination during transformation).

#### 20                   Example 2: Fermentation Protocol

A 250-fermentation (carried out in a 250-liter New Brunswick fermentor) was started in a 100-liter volume containing 67 liters of 10X basal salts [52 ml/L 85% phosphoric acid, 1.8 g/L calcium sulfate·2H<sub>2</sub>O, 28.6 g/L  
25 potassium sulfate, 23.4 g/L magnesium sulfate·7H<sub>2</sub>O, 6.5 g/L potassium hydroxide] and 3.6 Kg of glycerol. After

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sterilization, 420 ml of PTM<sub>1</sub> trace salts solution [6.0 g/L cupric sulfate·5H<sub>2</sub>O, 0.08 g/L sodium iodide, 3.0 g/L manganese sulfate·H<sub>2</sub>O, 0.2 g/L sodium molybdate·2H<sub>2</sub>O, 0.02 g/L boric acid, 0.05 g/L cobalt chloride, 56.0 g/L ferrous sulfate·7H<sub>2</sub>O, 0.2 g/L biotin and 5.0 ml/L sulfuric acid (conc)] were added and the pH was adjusted and subsequently controlled at 5.0 with the addition of ammonium hydroxide throughout the fermentation. Excessive foaming was controlled with the addition of 5% Struktol J673 antifoam.

10 The fermentor was inoculated with 1.5 liters of an overnight culture (OD<sub>600</sub> = 1:1 of the EGF-expressing strain of P. pastoris, G+EGF819S4 in YNB [6.7 g/l of yeast nitrogen base without amino acids (Difco, Detroit, MI)], 2% glycerol, 0.1 M phosphate, pH 6. The dissolved oxygen was

15 maintained above 20% by increasing, as appropriate the air flow rate up to a maximum value of about 200 liter/minute, the agitation up to a maximum value of about 300 rpm, the pressure of the fermentor up to a maximum value of about 10 psig, and/or by enriching the air sparge to the fermentor

20 with oxygen during the fermentation.

After exhaustion of the initial glycerol charge, a 50% glycerol feed, containing 12 ml/L PTM<sub>1</sub> trace salts, was initiated at a rate of 2 l/h; the glycerol feed continued for 7 hours, at which time the methanol feed,

25 containing 12 ml/L PTM<sub>1</sub> trace salts, was started at a rate of 0.24 Kg/h. The methanol feed was increased by about 10% each hour at half hour intervals until a methanol feed rate of about 1 Kg/h was reached. The fermentor contents were



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then harvested after about 44 hours of growth on methanol-containing feed.

### Example 3: Analytical HPLC

A. Reverse phase HPLC was performed with an analytical HPLC column; a Waters Bondapak C18 (0.25 X 30 cm) column with a C18 guard column using a Waters Model 600 Solvent Delivery System and Waters Intelligent Sample Processor Model 712 with refrigeration were used. Mobile Phase A consisted of 0.1% trifluoroacetic acid (TFA), i.e., 0.6 ml of 100% TFA per liter and Mobile Phase B was 95% acetonitrile/5% H<sub>2</sub>O with 0.1% TFA. The flow rate was 1.0 ml/minute. The column was equilibrated in 80% A and 20% B for 20 minutes before each run.

Each analytical run was 50 minutes. The first five minutes were isocratic at 80% A, 20% B; then a linear gradient over the next 25 minutes brought the composition to 70% A, 30% B; and, finally, the concentration of B was increased to 55% by a linear gradient during the final 20 minutes. UV absorbance was monitored at 210 nm with a Waters detector model 481, and recorded on a Shimadzu C-R3A integrator.

B. A shorter analytical HPLC procedure was developed for process control at the pilot scale. The shorter procedure consisted of a ten minute run at isocratic conditions of 72% A, 28% B.

Cells were removed from broth samples by centrifugation for three minutes in a microcentrifuge

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before the samples were loaded onto the HPLC.

**Example 4: Purification Protocols at 250-Liter Scale**

Human EGF-containing broth was separated from cells by centrifugation at a 3-liter per minute feed rate and 40 second shoot time (i.e., 40 second intervals between discharges) in an Alfa-Laval BTPX205 continuous centrifuge at approximately 13,000 xg. The cell cream was diluted with deionized water to its original volume and centrifuged as before. The clarified broths from the two separations were combined and further clarified by centrifuging again at a 6 liter per minute feed rate with a 20 minute shoot time.

Human EGF was removed from the resulting broth by stepwise addition of a reverse phase resin. Two aliquots of 200 g each, and subsequent aliquots of 300 g each, of Vydac 281TPB 15-20 wetted in methanol were added to the broth and the mix was stirred for 15 minutes after each addition. The amount of EGF remaining in the broth was measured by the shorter analytical procedure HPLC (as described in Example 3B) subsequent to each resin addition. Additional aliquots of resin were added until less than 10% of the starting EGF value was still present in the broth. In total, 1600 g of resin were added.

The resin was removed from the broth by pumping the resin-broth mixture through a column (30-cm diameter, Amicon) with a 10 mesh screen on the bottom support, and with the top screen removed. After the broth was passed

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through the column, the top screen was replaced, and the resin washed with about 5 liters of 0.05 M acetic acid. The EGF was then eluted with two 4-liter aliquots of 38% ethanol, and acidified to pH 3.5 with 0.05 M acetic acid.

5 The eluate was decolorized by loading said eluate onto a column of cation exchange resin at a ratio of not more than 25 g EGF per six liters of cation exchange resin (Macroorb KAX-CM Resin, Sterling Organics). The column had previously been activated/regenerated by washing with 1.0

10 N sodium acetate, then contacting with 0.1 N sodium hydroxide for about 1 hour at room temperature, followed by washing with 0.2 M acetic acid. The column was then equilibrated in 50 mM acetic acid. The EGF was then eluted from the column with 12 liters 0.3 M ammonium acetate

15 (having a conductivity of about 19,000 micromhos).

Aliquots of the eluate from the cation exchange column were loaded onto a preparative HPLC (Waters Delta prep, Model 3000) having a two inch diameter radial compression Waters C18 column, up to a loading level of

20 about 14 mg of EGF per  $\text{cm}^3$  of reverse phase resin. The column used was a Waters Prep Pak cartridge packed with Vydac C<sub>18</sub> reverse phase resin (having a 15-20 particle size and approximately 300A pore size). The column was first washed with about 500 ml of a mixture comprising 90% A, 10%

25 B at a flow rate of about 50 ml/min; EGF was then eluted in a 40-minute linear gradient to 25% B. Solvents A and B were those described above for analytical HPLC (see Example 3). Samples were collected in 40-ml aliquots, beginning

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about 15 minutes after the linear gradient was started, and continuing for a period of about 15 minutes. The EGF purity of each sample collected was assessed by analytical HPLC (see Example 3). The samples were pooled to give a  
5 final purity greater than 95%.

To remove acetonitrile and TFA from the EGF-containing fraction, the pooled fractions were loaded onto a 6-liter cation exchange column (Macrosorb KAX-CM resin), and washed with 0.05 M acetic acid until the acetonitrile  
10 concentration was below 10 ppm. EGF was then eluted with 0.3 M ammonium acetate. The eluate was filtered through a 0.2 filter and lyophilized to dryness. The recovery of EGF obtained at each step of the invention process is summarized in Table I.

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TABLE I  
RECOVERY AND PURIFICATION OF EGF  
FROM EGF-CONTAINING BROTH

5	<u>Sample</u>	<u>Volume (L)</u>	<u>EGF Concentra- tion (g/L)</u>	<u>Total EGF (g)</u>	<u>Recovery Per Step (%)</u>	<u>Overall Recovery (%)</u>
10	Clarified Broth & Wash	210	0.225	47	--	100
	Bulk resin eluate	8	4.90	38.9	83	83
	Decolor- ization	15	2.60	39.4	101	84
15	Preparative HPLC	1	19.00	37.5	95	80
	Acetonitrile Removal	15	20.00	30	80	64
20	Sterile Filtration	15	1.83	27.5	92	58
	Lyophili- zation	--	--	27.5	98	57

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The results summarized in Table I demonstrate that the invention process provides a very efficient means to purify large volumes of EGF-containing medium. Greater than 50% recovery of EGF having a purity of at least 95% is  
5 achieved.

The invention has been described in detail with respect to certain particular embodiments thereof, but reasonable variations and modifications, within the spirit and scope of the present disclosure, are contemplated by  
10 the present disclosure and the appended claims.

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THAT WHICH IS CLAIMED IS:

1. Method for the purification of epidermal growth factor (EGF) peptides from medium containing EGF, said method comprising:

5 (a) contacting said medium with a sufficient quantity of a reverse phase resin and under conditions suitable to adsorb at least 90% of said EGF from said medium,

(b) eluting the adsorbed EGF from said EGF-  
10 containing resin of step (a) by contacting said resin with a sufficient quantity of a solvent system which is sufficiently non-polar so as to displace substantially all of said EGF from said resin,

(c) contacting the eluate from step (b) with a  
15 sufficient quantity of a cation exchange resin and under conditions suitable to adsorb at least 95% of said EGF from said eluate,

(d) eluting the adsorbed EGF from said cation exchange resin by contacting said resin with at least 1.5  
20 volumes, relative to the volume of resin, of a buffer system having a sufficient ionic strength so as to displace substantially all of said EGF from said resin, and thereafter

(e) subjecting at least a portion of the eluted  
25 EGF obtained from step (d) to preparative-scale high performance liquid chromatography (HPLC).

2. A method in accordance with claim 1 wherein

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said medium contains at least 0.1 grams of EGF per liter of said medium.

3. A method in accordance with claim 2 wherein said medium from which EGF is purified is the fermentation  
5 broth from a fermentation operation.

4. A method in accordance with claim 3 wherein said medium from which EGF is purified is the fermentation broth from a high cell density fermentation operation.

5. A method in accordance with claim 4 wherein  
10 said medium from which EGF is purified is the fermentation broth from a high cell density yeast fermentation operation, wherein said yeast are transformed with at least one DNA fragment capable of expressing EGF.

6. A method in accordance with claim 5 wherein  
15 said yeast are selected from the genus Pichia.

7. A method in accordance with claim 5 wherein said yeast is P. pastoris strain G+EGF819S4.

8. A method in accordance with claim 5 wherein cellular and particulate material are separated from the  
20 fermentation broth prior to the contacting contemplated by step (a).



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9. A method in accordance with claim 1 wherein said reverse phase resin is selected from C<sub>8</sub>-C<sub>18</sub> resins, CN (cyano) resins, NH<sub>2</sub> (amino) resins or phenyl resins.

10. A method in accordance with claim 9 wherein  
5 said reverse phase resin is a C<sub>18</sub>-type resin.

11. A method in accordance with claim 10 wherein at least 30 grams, per gram of EGF in said medium, of said reverse phase resin are employed.

12. A method in accordance with claim 1 wherein  
10 the contacting contemplated by step (a) is carried out for a time in the range of about 0.1 up to 8 hours, at a temperature in the range of about 4 up to 40°C.

13. A method in accordance with claim 1 wherein said EGF-containing resin is separated from the EGF-  
15 depleted broth of step (a) prior to carrying out the elution contemplated by step (b).

14. A method in accordance with claim 13 wherein said separation is carried out by filtration, decantation, or centrifugation.

20 15. A method in accordance with claim 14 wherein said separation is carried out by filtration.

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16. A method in accordance with claim 12 wherein said contacting is carried out by passing the medium containing EGF through a column containing said reverse phase resin.

5           17. A method in accordance with claim 1 wherein, prior to step (b), said resin is contacted within the range of about 1 up to 10 volumes, relative to the volume of resin, of a dilute, weak acid, which dilute, weak acid is then removed from said EGF-containing resin.

10           18. A method in accordance with claim 17 wherein the dilute, weak acid, is selected from a 0.05 M acetic acid, formic acid or phosphoric acid solution.

15           19. A method in accordance with claim 1 wherein suitable solvents for the step (b) elution are selected from:

aqueous alcohol mixtures containing at least one alcohol having up to 4 carbon atoms,  
aqueous acetonitrile,  
ketones having up to 6 carbon atoms,  
20           cyclic ethers, or cyclic polyethers having up to 6 carbon atoms.

20           20. A method in accordance with claim 10 wherein the solvent employed for the step (b) elution is 38% aqueous ethanol.

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21. A method in accordance with claim 1 wherein said cation exchange resin is selected from carboxymethyl cellulose, carboxymethyl sephadex, sulphopropyl cellulose, or sulphopropyl sephadex.

5           22. A method in accordance with claim 21 wherein the contacting of partially purified broth with cation exchange resin is carried out for a contact time of at least 1 min and at a temperature in the range of about 4 up to 40°C.

10           23. A method in accordance with claim 22 wherein said cation exchange resin is carboxymethyl cellulose or carboxymethyl sephadex.

24. A method in accordance with claim 22 herein said cation exchange resin is carboxymethyl cellulose.

15           25. A method in accordance with claim 24 wherein said cation exchange resin is activated/regenerated by sequential contact with 1.0 N sodium acetate, 0.1 N sodium hydroxide, then 0.2 M acetic acid.

20           26. A method in accordance with claim 24 wherein the quantity of cation exchange resin employed falls in the range of about 0.25 up to 1 liter per gram of EGF in said eluate.

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27. A method in accordance with claim 1 wherein the EGF-containing resin produced in step (c) is contacted with a sufficient quantity of a dilute, weak acid solution to reduce the absorbance, at 400 nanometers, of the effluent from said contacting to 0.1 A.U. or less, relative to a blank sample of the dilute, weak acid solution.

28. A method in accordance with claim 27 wherein the dilute, weak acid is selected from a 0.05 M acetic acid, formic acid or phosphoric acid solution.

29. A method in accordance with claim 1 wherein said buffer system having a sufficient ionic strength so as to displace substantially all of said EGF from said resin has an ionic strength of at least 0.1 g-ions/L and is selected from ammonium acetate, ammonium formate, sodium acetate, potassium acetate or sodium chloride.

30. A method in accordance with claim 29 wherein said buffer system having an ionic strength of at least 0.1 g-ions/L is 0.3 M ammonium acetate.

31. A method in accordance with claim 30 wherein, the elution of EGF from the cation exchange resin, in the range of about 1.5 up to 3 volumes, per volume of said resin, of said buffer system is employed.

32. A method in accordance with claim 1 wherein

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the pH of the eluted EGF obtained from step (d) is adjusted prior to step (e) by the addition thereto of a sufficient quantity of trifluoroacetic acid (TFA) to render said solution about 0.1% in TFA.

5           33. A method in accordance with claim 1 wherein the HPLC column onto which the EGF-containing solution is loaded is selected from C<sub>8</sub>-C<sub>18</sub> resins, CN (cyano) resins, NH<sub>2</sub> (amino) resins or phenyl resins.

          34. A method in accordance with claim 1 wherein  
10 said reverse phase resin is a C<sub>18</sub>-type resin.

          35. A method in accordance with claim 34 wherein the initial solvent system employed in the HPLC step is sufficiently non-polar to elute impurities less hydrophobic than EGF, but not so non-polar as to cause elution of  
15 significant amounts of EGF.

          36. A method in accordance with claim 35 wherein said initial solvent system comprises:

          a 0.1% trifluoroacetic acid-containing aqueous solution, and

20           up to 15% of a 95% acetonitrile-5% water mixture containing 0.1% TFA.

          37. A method in accordance with claim 33 wherein the solvent system employed for the elution of EGF from the

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HPLC is a mixed solvent system comprising an aqueous component and an organic component, wherein said mixed solvent system is sufficiently non-polar to cause elution of EGF, but not so non-polar as to cause elution of significant quantities of materials which are more tightly bound to the HPLC support than is EGF.

38. A method in accordance with claim 37 wherein the elution of EGF from the HPLC is carried out by gradually increasing the proportion of the organic component of said mixed solvent system, relative to the proportion of the aqueous component of the mixed solvent system, during the course of the elution.

39. A method in accordance with claim 38 wherein the solvent system comprises:

an initial concentration of up to 100%, down to a final concentration as low as 50%, of a 0.1% trifluoroacetic acid-containing aqueous solution, and

an initial concentration as low as 0%, up to a final concentration of up to 50%, of an aqueous acetonitrile mixture containing 0.1% TFA, wherein said aqueous acetonitrile contains up to 20% water.

40. A method in accordance with claim 39 further comprising the steps:

(f) contacting the eluate from step (e) with a sufficient quantity of a cation exchange resin and under

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conditions suitable to adsorb at least 95% of said EGF from said eluate,

(g) contacting the EGF-containing resin produced in step (f) with sufficient quantity of a dilute, weak acid solution to reduce the concentration of acetonitrile to no greater than about 10 mg/L, and

(h) eluting the adsorbed EGF from said cation exchange resin by contacting said resin with at least 1.5 volumes, relative to the volume of resin, of a buffer system having a sufficient ionic strength so as to displace substantially all of said EGF from said resin.

41. A method in accordance with claim 40 wherein said cation exchange resin is carboxymethyl cellulose.

42. A method in accordance with claim 40 wherein the dilute, weak acid is selected from a 0.05 M acetic acid, formic acid or phosphoric acid solution.

43. A method in accordance with claim 40 wherein said buffer system having a sufficient ionic strength so as to displace substantially all of said EGF from said resin is 0.3 M ammonium acetate.

44. A method in accordance with claim 40 wherein the eluate of step (h) is lyophilized to dryness.

45. A method in accordance with claim 44

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wherein, prior to being lyophilized, the eluate of step (g) is filtered through a pore filter having a pore size no greater than 0.45 microns.

46. A method in accordance with claim 1 wherein  
5 the epidermal growth factor is selected from native human epidermal growth factor (1-53 EGF), (1-48) analog of hEGF, (1-51) analog of hEGF, (1-52) analog of hEGF, as well as peptides which are substantially homologous thereto.

47. Method for the purification of human  
10 epidermal growth factor (hEGF) peptides from medium containing hEGF, wherein said medium containing hEGF is the fermentation broth from a high cell density yeast fermentation operation, and wherein said yeast are transformed with at least one DNA fragment capable of  
15 expressing hEGF, said method comprising:

(a) contacting said medium with a sufficient quantity of a reverse phase resin and under conditions suitable to adsorb at least 90% of said hEGF from said medium; wherein said reverse phase resin is C<sub>18</sub>-type resin;  
20 wherein at least 30 grams, per gram of hEGF in said medium, of said reverse phase resin are employed; and wherein said contacting is carried out for a time in the range of about 0.1 up to 8 hours, at a temperature in the range of about 4 up to 40°C,

25 (b) separating the hEGF-containing resin from the hEGF-depleted medium,



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(c) contacting the hEGF-containing resin with at least 1 volume, per volume of resin, of a dilute, weak acid; wherein the dilute, weak acid is a 0.05 M acetic acid solution, then removing the dilute, weak acid from the  
5 hEGF-containing resin,

(d) eluting the adsorbed hEGF from said hEGF-containing resin of step (c) by contacting said resin with a sufficient quantity of a solvent system which is sufficiently non-polar so as to displace substantially all  
10 of said hEGF from said resin,

(e) contacting the eluate from step (d) with a sufficient quantity of a cation exchange resin and under conditions suitable to adsorb at least 95% of said hEGF from said eluate,

15 (f) contacting the hEGF-containing resin produced in step (e) with sufficient quantity of a dilute, weak acid solution to reduce the absorbance, at 400 nanometers, of the effluent from said contacting to 0.1 A.U. or less, relative to a blank sample of the dilute,  
20 weak acid solution,

(g) eluting the adsorbed hEGF from said cation exchange resin by contacting said resin within the range of about 1.5 up to 3 volumes, relative to the volume of resin, of a buffer system comprising 0.3 M ammonium acetate,

25 (h) adjusting the pH of the eluted hEGF obtained from step (g) by the addition thereof of a sufficient quantity of trifluoroacetic acid to render said solution about 0.1% in TFA,

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- (i) loading at least a portion of the eluted, pH-adjusted hEGF obtained from step (h) onto a preparative-scale high performance liquid chromatography column (HPLC),
- (j) initially treating the loaded column within
- 5 the range of about 1 up to 2 column volumes of a solvent system which is sufficiently non-polar to elute impurities less hydrophobic than hEGF, but not so non-polar as to cause elution of significant amounts of hEGF, and thereafter
- 10 (k) eluting purified hEGF from said HPLC employing a mixed solvent system comprising an aqueous component and an organic component, wherein said mixed solvent system is sufficiently non-polar to elute hEGF, but not so non-polar as to cause elution of significant
- 15 quantities of materials which are more tightly bound to the HPLC support than is hEGF.

48. A method in accordance with claim 47 wherein the solvent system employed for step (j) comprises:

90% of a 0.1% trifluoroacetic acid-containing

20 aqueous solution, and

10% of a 95% acetonitrile-5% water mixture containing 0.1% TFA.

49. A method in accordance with claim 47 wherein the mixed solvent system employed for elution of hEGF from

25 said HPLC comprises:

an initial concentration of 90%, down to a final

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concentration of 75%, of a 0.1% trifluoroacetic acid-containing aqueous solution, and

an initial concentration of 10%, up to a final concentration of 25%, of a 95% acetonitrile-5% water mixture containing 0.1% TFA.

50. A method in accordance with claim 47 further comprising the steps:

(l) contacting the eluate from step (k) with a sufficient quantity of a cation exchange resin and under conditions suitable to adsorb at least 95% of said hEGF from said eluate,

(m) contacting the hEGF-containing resin produced in step (l) with sufficient quantity of a dilute, weak acid solution to reduce the concentration of acetonitrile in the effluent to no greater than about 10 mg/L, and thereafter

(n) eluting the adsorbed hEGF from said cation exchange resin by contacting said resin with at least 1.5 volumes, relative to the volume of resin, of a solvent system having an ionic strength of about 0.3 g-ions/L.

51. A method in accordance with claim 50 wherein said cation exchange resin is carboxymethyl cellulose.

52. A method in accordance with claim 50 wherein the dilute, weak acid is a 0.05 M acetic acid solution.

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53. A method in accordance with claim 52 wherein said cation exchange resin is activated/regenerated by sequential contact with 1.0 N sodium acetate, 0.1 N sodium hydroxide, then 0.2 M acetic acid.

5 54. A method in accordance with claim 50 wherein said buffer system is 0.3 M ammonium acetate.

55. A method in accordance with claim 50 wherein the eluate of step (h) is lyophilized to dryness.

56. A method in accordance with claim 55  
10 wherein, prior to being lyophilized, the eluate of step (g) is filtered through a pore filter having a pore size no greater than about 0.45 microns.

**AMENDED CLAIMS**

[received by the International Bureau  
on 29 July 1991 (29.07.91);  
original claims 3-5 cancelled; original claims 1,6-8 and 47  
amended; other claims unchanged (4 pages)]

1. Method for the purification of epidermal growth factor (hEGF) peptides from medium containing human hEGF, said method comprising:

5 (a) contacting said medium with a sufficient quantity of a reverse phase resin and under conditions suitable to adsorb at least 90% of said hEGF on said reverse phase resin from said medium,

(b) eluting the adsorbed EGF from said EGF-  
10 containing resin of step (a) by contacting said resin with a sufficient quantity of a solvent system which is sufficiently non-polar so as to displace substantially all of said hEGF from said resin,

(c) contacting the eluate from step (b) with a  
15 sufficient quantity of a cation exchange resin and under conditions suitable to adsorb at least 95% of said hEGF from said eluate,

(d) eluting the adsorbed hEGF from said cation exchange resin by contacting said resin with at least 1.5  
20 volumes, relative to the volume of resin, of a buffer system having a sufficient ionic strength so as to displace substantially all of said hEGF from said resin, and thereafter

(e) subjecting at least a portion of the eluted  
25 hEGF obtained from step (d) to preparative-scale high performance liquid chromatography (HPLC),

(f) initially treating the loaded column within the range of about 1 up to 2 column volumes of a solvent

system which is sufficiently non-polar to elute impurities less hydrophobic than hEGF, but not so non-polar as to cause elution of significant amounts of hEGF, and thereafter

- 5 (g) eluting purified hEGF from said HPLC employing a mixed solvent system comprising an aqueous component and an organic component, wherein said mixed solvent system is sufficiently non-polar to elute hEGF.

2. A method in accordance with claim 1 wherein  
10 said medium contains at least 0.1 grams of EGF per liter of said medium.

6. A method in accordance with claim 1 wherein said yeast are selected from the genus Pichia.

7. A method in accordance with claim 1 wherein  
15 said yeast is P. pastoris strain G+EGF819S4.

8. A method in accordance with claim 1 wherein cellular and particulate material are separated from the fermentation broth prior to the contacting contemplated by step (a).

wherein, prior to being lyophilized, the eluate of step (g) is filtered through a pore filter having a pore size no greater than 0.45 microns.

46. A method in accordance with claim 1 wherein  
5 the epidermal growth factor is selected from native human epidermal growth factor (1-53 EGF), (1-48) analog of hEGF, (1-51) analog of hEGF, (1-52) analog of hEGF, as well as peptides which are substantially homologous thereto.

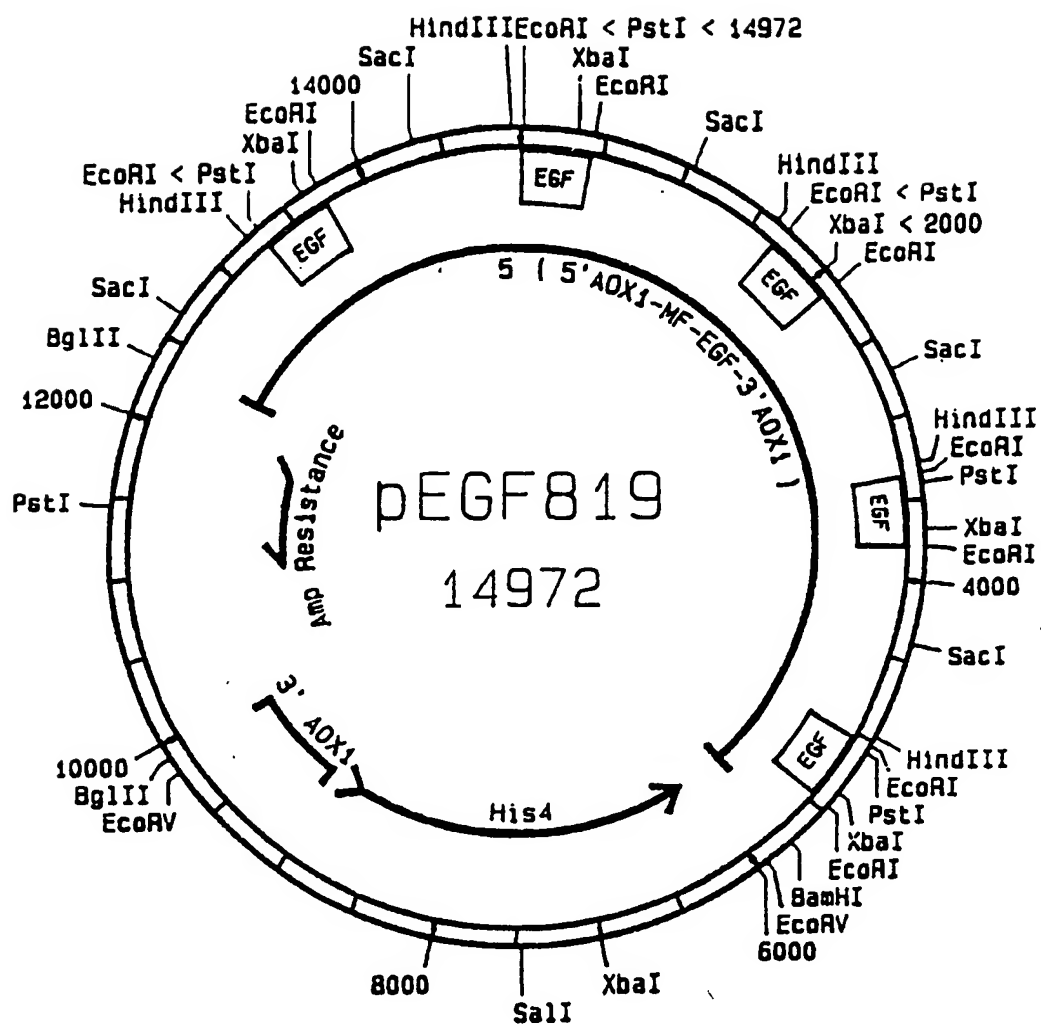
47. Method for the purification of human  
10 epidermal growth factor (hEGF) peptides from medium containing hEGF, wherein said medium containing hEGF is the fermentation broth from a high cell density yeast fermentation operation, and wherein said yeast are transformed with at least one DNA fragment capable of  
15 expressing hEGF, said method comprising:

(a) contacting said medium with a sufficient quantity of a reverse phase resin and under conditions suitable to adsorb at least 90% of said hEGF on said reverse phase resin and to thereby separate said hEGF from  
20 said medium; wherein said reverse phase resin is C<sub>18</sub>-type resin; wherein at least 30 grams, per gram of hEGF in said medium, of said reverse phase resin are employed; and wherein said contacting is carried out for a time in the range of about 0.1 up to 8 hours, at a temperature in the  
25 range of about 4 up to 40°C,

(b) separating the hEGF-containing resin from  
the hEGF-depleted medium,



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## INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/02551

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all *)		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC(5): A 61K 37/02; C07K 1/00		
U.S. CL.: 530/300, 324, 344, 417, 824		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched *		
Classification System	Classification Symbols	
U.S. CL.	530/300, 324, 344, 417, 824	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *		
AFS DATABASE CAS DATABASE		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT *</b>		
Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages †	Relevant to Claim No. ‡
Y	CHEMICAL ABSTRACTS, VOLUME 104, ISSUED 1986, TVASHCHENKO ET AL., "RAPID ISOLATION OF HIGHLY PURIFIED EPIDERMAL GROWTH FACTOR BY REVERSE-PHASE LIQUID CHROMATOGRAPHY", SEE ABSTRACT NO. 622249., EKSP. ONKOL., 7 (6), 47-9.	1-56
Y	BIOTECHNIQUES, ISSUED NOV/DEC 1983, SOFER ET AL., "DESIGNING AN OPTIMAL CHROMATOGRAPHIC PURIFICATION SCHEME FOR PROTEINS", PAGES 198-203, SEE PAGES 198, 200.	1-56
Y	CHEMICAL ABSTRACTS, VOLUME 103, ISSUED 1985, NISHIMURO ET AL., "HETEROGENEITY OF HUMAN EPIDERMAL GROWTH FACTOR/UROGASTRONE FROM HUMAN URINE", SEE ABSTRACT NO. 207578K CHEM. PHARM. BULL., 33(9), 4037-40.	1-56
<p>* Special categories of cited documents: †</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (is specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but used to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"Z" document number of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search		Date of Mailing of the International Search Report
28 MAY 1991		25 JUN 1991
International Searching Authority		Examiner
ISA/US		Bennett Celser BENNETT CELSA

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y

CHEMICAL ABSTRACTS, VOLUME 98,  
ISSUED 1983, OHARE ET AL.,  
"THE OPTIMIZATION OF RP- HPLC OF  
PROTEINS WITH LARGE PORE-SIZE SHORT  
ALKYLCHAIN-BONDED SILICA (ULTRAPORE RPSC)  
AND ITS APPLICATION TO EPIDERMAL GROWTH  
FACTOR", SEE ABSTRACT NO. 19185; PROTIDES  
BIOLOGICAL FLUIDS, 30, 723-6.

4-8,47-56

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE<sup>1</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ..... because they relate to subject matter<sup>1</sup> not required to be searched by this Authority, namely:
  
2. ☐ Claim numbers ..... because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out<sup>1</sup>, specifically:
  
3. ☐ Claim numbers ..... because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING<sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
  
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
  
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.  
☐ No protest accompanied the payment of additional search fees.